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Daniel M. Sullivan
Examiner AU 1636
Room: 12D12
Mail Box: 11E12
Tel: 703-305-4448

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Conditional mutagenesis in mice: the Cre/loxP recombination system*

A. PLÜCK

European Molecular Biology Laboratory, Cell Regulation Programme, Meyerhofstr. 1, D-69012 Heidelberg, Federal Republic of Germany

Simple loss-of-function mutations often cannot reveal the multiple roles of genes in different tissues and at different times during development. Conditional mutagenesis, such as gene disruption limited to certain cell lineages or to certain stages during development, may allow a detailed analysis of gene function.

Conditional gene targeting in mice is achieved by a combination of homologous recombination and site specific recombination. First, the recognition sites for the site specific recombinase are introduced in the desired position in the genome of murine ES cells by homologous recombination. In a second step site-specific recombination is catalysed by expression of the corresponding recombinase. Homologous recombination relies on the cell's endogenous recombinational machinery. In contrast, for site specific recombination a recombinase is supplied exogenously. This review focuses on the use of the Cre/loxP recombination system for the generation of gene targeted mice.

The Cre/loxP site specific recombinase system

Site specific recombinases (SSR) are enzymes encoded by bacterial and yeast elements that recognize specific target sites at which DNA is cleaved and re-ligated to induce recombination. The 38 kDa Cre (causes recombination) recombinase from bacteriophage P1 belongs to the integrase family of recombinases. It catalyses site specific recombination between specific DNA target sites of 34 bp each termed loxP (locus of crossing over) (Sternberg & Hamilton 1981). In the bacteriophage P1, Cre ensures maintenance of the plasmid by resolving DNA dimers into plasmid monomers. Cre itself is sufficient to catalyse recombination between loxP sites; no additional cofactor is needed. This feature makes the Cre/loxP recombination system a useful tool for genetic engineering and allows the application of the system in other species (Kilby *et al.* 1993). Each loxP site consists of two inverted repeats of 13 bp flanking an asymmetric spacer region of 8 bp which defines the orientation. Recombination between two sites occurs with base pair precision and is achieved by an assembly of four subunits of the recombinase: two subunits bind to the inverted repeats in each of the recombining elements and Cre synapses the binding sites and cleaves the DNA in the spacer region. A Holliday intermediate is formed which is then resolved. The Cre induced DNA rearrangement is a reversible reaction, such that intramolecular excision is favoured over integration and the extent of recombination is proportional to the level of recombinase expression. Depending on the orientation of the loxP sites to each other and whether they are located cis or trans, recombination results in different DNA rearrangements as depicted in Figure 1.

Applications of the Cre/loxP site specific recombinase system in gene targeting

Cre mediated recombination, providing Cre activity by transient transfection of ES cells, can be used in a number of different applications after introducing the specific target sites by homologous recombination into the locus of interest. For example, this strategy has been used to remove a loxP flanked selection marker gene from the locus. In addition, a limited stretch of DNA can also be deleted (Gu *et al.* 1993).

The introduction of new gene fragments together with two loxP sites into the gene of interest makes gene replacement possible utilizing a two-step gene targeting procedure. Large chromosomal deletions (3–4cM) have been performed after introducing two loxP sites on the same chromosome in direct repeat orientation by two independent homologous recombination events (Ramirez-Solis *et al.* 1995).

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Site specific recombination has recently also been applied to introduce intermolecular recombination events after placing two loxP sites on different chromosomes by sequential homologous recombination events (Smith *et al.* 1995; Van Deursen *et al.* 1995).

In a transient transgenesis approach, pronuclear microinjection of a circular plasmid encoding the Cre recombinase into fertilized oocytes of mice, can be used to excise a reporter transgene flanked by loxP sites (Araki *et al.* 1995).

Cell type-specific gene targeting

Cre mediated recombination can be activated *in vivo* by using a mouse strain expressing the recombinase. Mutations can be introduced in a cell type specific

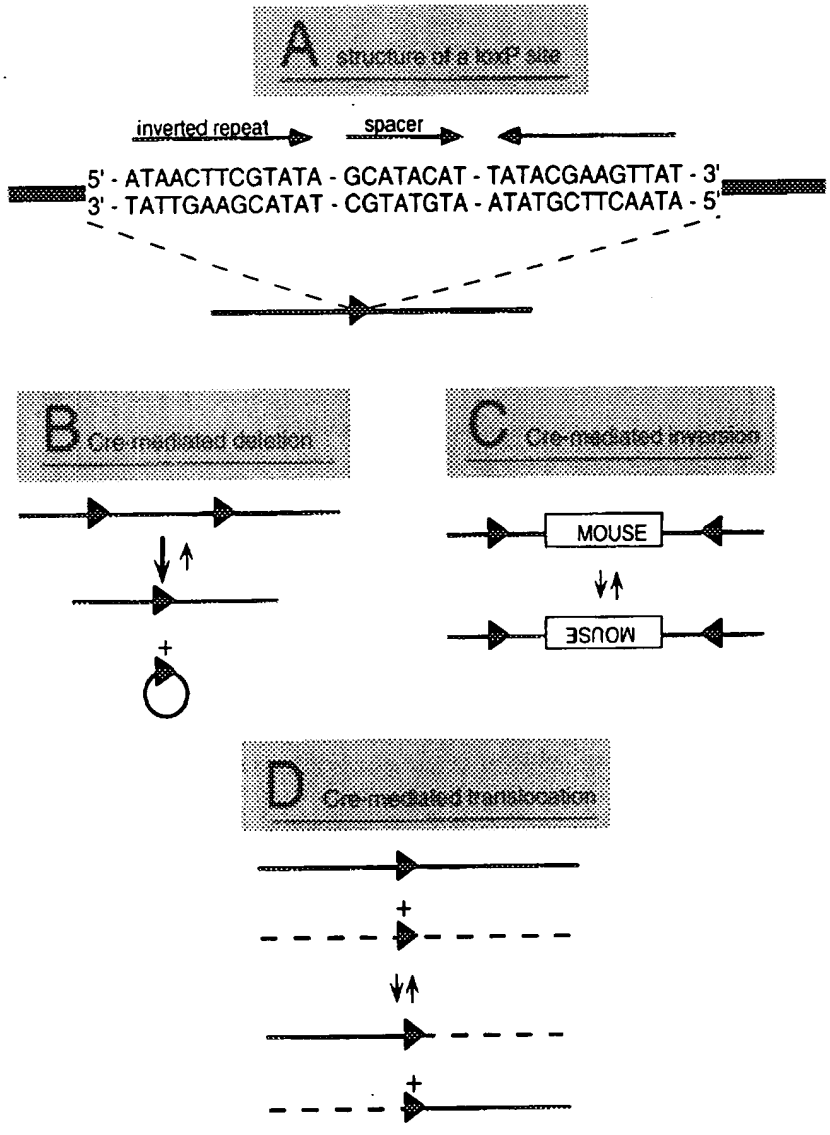

Figure 1. The Cre/loxP recombination system. A, Structure of a loxP site and B–D, recombination products of intra and intermolecular Cre mediated rearrangements. A, A 34 bp loxP site consisting of two 13 bp inverted repeats and an asymmetric 8 bp spacer region which defines the orientation of the loxP site symbolized by a black triangle. B, Cre mediated recombination between two head to tail oriented loxP sites in cis leads to the excision of the intervening sequence as a circular molecule. One intact loxP site remains on each of the recombination products. C, Cre mediated recombination leads to inversion of a loxP flanked DNA segment if loxP sites are in opposite orientation in cis. D, Cre-mediated translocation by reciprocal exchange of DNA segments flanking two parallel oriented sites in trans.

and/or inducible fashion leading to conditional gene inactivation or conditional reconstitution of gene function ('gene repair').

As shown in Figure 2, for a cell type-specific gene inactivation (Gu *et al.* 1994) two mouse strains are required: one which carries a floxed (loxP flanked) allele for the target gene, and another which expresses Cre in a cell type-specific fashion. In offspring derived from an intercross of these two strains, cell type-specific recombination will occur in cells where Cre is expressed. The target gene is thus deleted in a restricted fashion and should remain functional in all cells where the Cre gene is not expressed.

Gene targeting 'flox and delete' strategy

The first type of mouse strain is generated by homologous recombination in ES cells using the so-called 'flox and delete' strategy illustrated in Figure 3. In this strategy a part of the target gene is flanked by loxP sites introduced by homologous recombination in ES cells. The amount of expression from the floxed allele should be the same compared to the wildtype allele. The targeting vector is designed so that the part in the gene to be deleted is preceded by one loxP site (as part of the region of homology). The selection marker gene flanked by two loxP sites is then introduced downstream of the sequence to be deleted. Finally, the targeting vector contains three loxP sites in a direct repeat configuration. After homologous recombination this strategy allows for an additional *in vitro* modification step by transient transfection of Cre (as described previously).

As a result of this transfection three types of recombination products *in vitro* can be produced. The type I recombination product resembles a recombination event between the two most distant loxP sites resulting in a full deletion. These ES cells can be used to create a null mutant mouse. The type II recombination product resembles a recombination between the two loxP sites flanking the selectable marker gene. The gene locus flanked by two loxP sites remains intact. If these ES cells are used for the generation of mice, the loxP flanked locus can later be inactivated in a conditional manner when the mouse is crossed to a relevant recombinase transgenic mouse.

In the third type of recombination product a gene segment is deleted leaving the selection marker gene intact. This type of ES cell will not be found, because they will be susceptible to negative selection.

Cre transgenic mice

Cre transgenic mice can be produced either by conventional pronuclear microinjection of a linearized, randomly

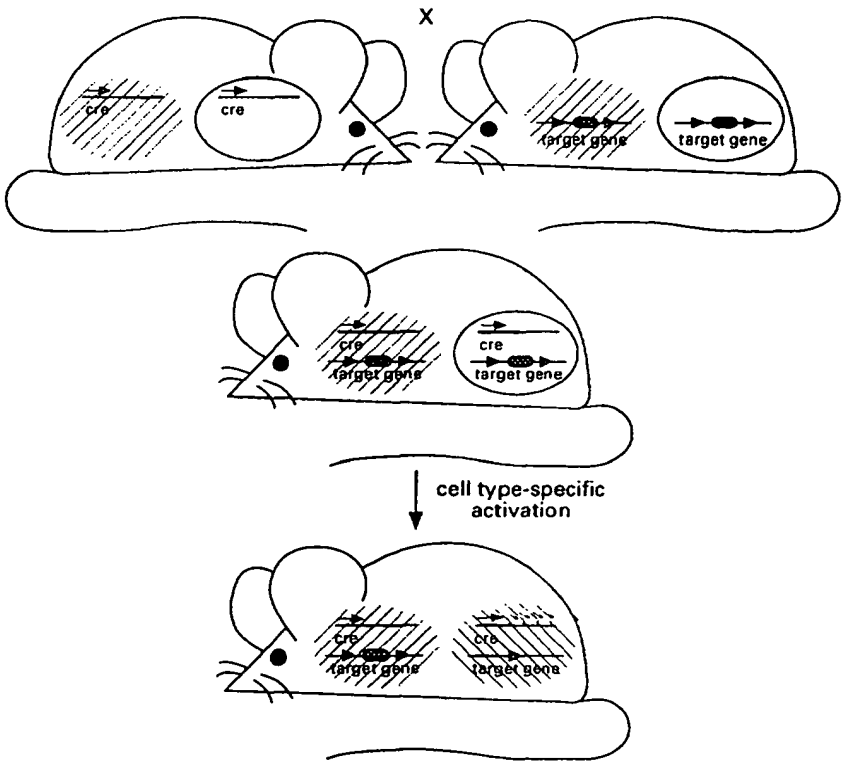

Figure 2. Breeding scheme for the approach of cell type specific gene targeting by homologous and site specific recombination. Only one allele of the target gene is shown. Two independent mouse strains have to be generated. One mouse strain should express the recombinase in a regulated fashion and can be generated by either conventional transgenesis or targeted insertion of the Cre gene into a locus of interest. Cells which will not express Cre are represented by ▨ filled circle, cells that will express Cre are shown as open white circles. The second mouse strain carries the gene of interest flanked by loxP sites in all cells. After intercross of the two strains cell type-specific activation of the recombinase (small arrows) should induce recombination in a particular set of cells, indicated by the ▧ filled circle.

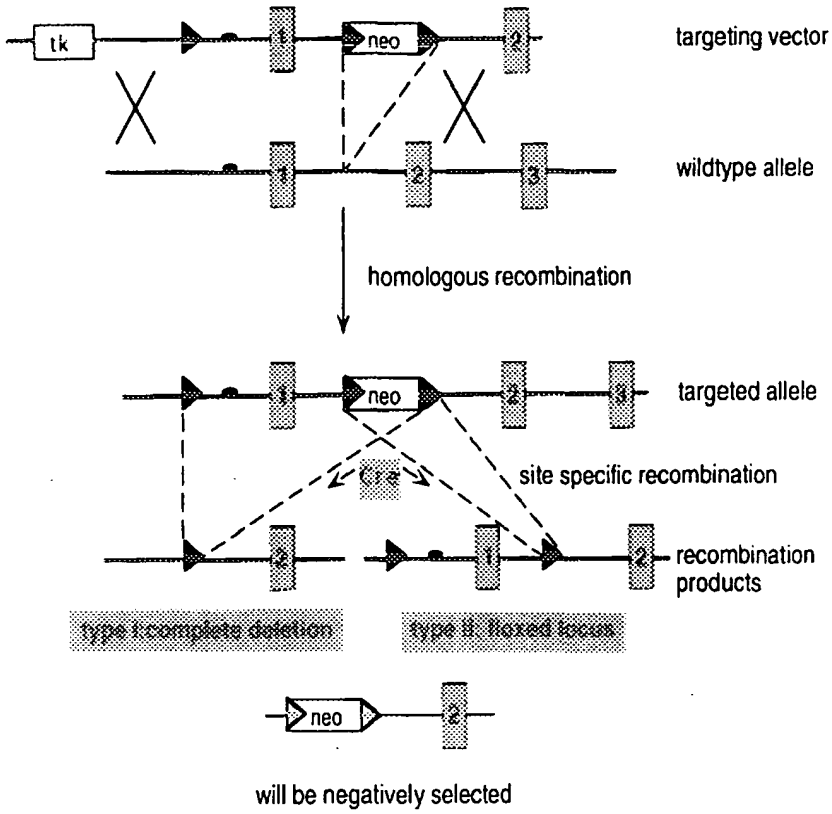

Figure 3. Gene targeting ('flox and delete') strategy for the simultaneous generation of a loxP flanked ('floxed') allele (still active) and a null allele of a gene locus in ES cells. In this case the loxP flanked selectable marker gene (neo) is placed in the intron between the two first exons of the target gene. An additional loxP site is introduced upstream of the promoter region in the targeting vector. All three lox P sites are oriented in head to tail configuration. Depending on which pair of loxP sites is activated for site specific recombination after transient transfection of Cre in the targeted ES cell clone, either the type I or the type II product is generated. The type I recombination product resembles a recombination between the two most distant loxP sites which deletes a DNA segment essential for gene function. The type II recombination product resembles a recombination between the two loxP sites flanking the resistance marker gene generating a gene locus with loxP flanked promoter region and first exon. The third type of recombination product where the target gene is inactivated but the neo gene is still intact will be negatively selected during the screening of G418 containing medium.

integrating expression vector into fertilized oocytes or as Cre 'knock-in' mice by targeted insertion of the Cre gene in frame into the coding region of a gene of interest.

An expression vector for the generation of conventional transgenic mice is shown in Figure 4. A fully characterized and tested promoter region from a gene with a cell type-specific regulation can be used to drive the expression of the Cre recombinase. Since the Cre gene does not itself contain any intron sequences, a splice donor and acceptor site and a polyadenylation signal sequence have to be provided in the vector. Since transgene integration occurs randomly (the number of transgene copies and the integration site are important parameters) in the genome, the expression pattern of the Cre recombinase often varies greatly.

Thus all the transgenic founders generated have to be screened for the desired expression pattern of the recombinase by crossing them to a reporter mouse strain carrying a reporter transgene as a single copy gene driven by a universally expressed promoter. The transgene can be expressed only if an inhibitory sequence flanked by loxP sites is removed after Cre expression. If the lacZ gene is used as a reporter gene the expression pattern of the Cre recombinase can then be analysed either at the DNA level by Southern blot detection or at the level of single cells by detecting β-galactosidase activity in histological sections. A transgene for a Cre reporter mouse is shown in Figure 5. Unfortunately, the ideal reporter mouse expressing the reporter gene under the control of a universal promoter which could be switched on in any cell after Cre expression is still not available. Most of the strains provided at present can be used only with restrictions for certain developmental stages or tissues. Two experimental approaches should soon be successful: one is to screen a large number of transgenic mice generated by introducing the transgene via electroporation of ES cells. This has two advantages: first, after electroporation only one or a low copy number of the transgene is integrated. Second, ES cells can be screened *in vitro* for expression of the reporter gene after induction of the recombination. For this purpose, for example, modifications of the β-actin promoter are tested. The second approach is to use one of the completely 'blue'

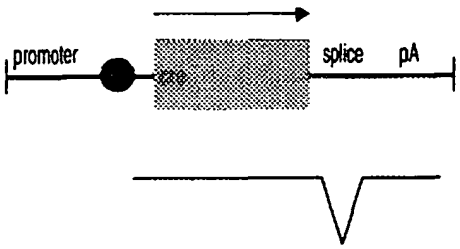

Figure 4. Cre transgenic mice. A typical expression vector for the generation of transgenic mice by pronuclear microinjection is shown. A promoter region which has been characterized for the properly regulated expression of a reporter gene in transgenic mice, directs the 1 kb intronless coding region of the Cre recombinase (box). A splice donor and acceptor site are located in the 3′ untranslated region of the cre gene followed by a polyadenylation signal (pA). These elements should support efficient transgene expression. The resulting transcript is shown as a thin line below.

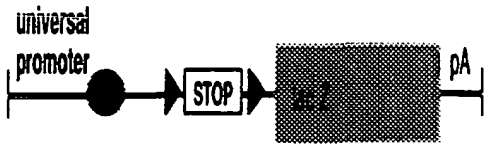

Figure 5. Transgenic Cre reporter lacZ mouse. Diagram of a lacZ reporter gene expression cassette. The lacZ reporter cassette consists of the promoterless lacZ coding region connected to a 5′ loxP flanked DNA fragment which is composed of strong transcriptional (pA) and translational (TAG) stop elements. This cassette is under the control of a universally active promoter. After deletion of the STOP elements by site directed mutagenesis, the lacZ gene should be expressed.

gene trap mouse lines (where the lacZ gene is expressed early in ontogeny in all tissues) and try to hit the same locus with the loxP flanked STOP version of the lacZ gene in ES cells.

As an alternative strategy the gene encoding the Cre recombinase can be introduced into the coding region of a gene regulated in the desired cell type specific way by homologous recombination in ES cells (Rickert *et al.* 1995) as depicted in Figure 6. For the removal of the selectable marker gene in this case it is necessary to use target sites for the site specific recombination of a different recombinase system, since the cell type specific activation of the Cre recombinase could potentially lead to chromosomal rearrangements after crossing with a target locus mouse containing additional loxP sites.

The recombinase FLP, derived from *S. cerevisiae*, also belongs to the integrase family and performs site specific recombination on DNA target sites termed FRT. FRT sites have a comparable structure to loxP sites. The generation of a non-functional allele in the target locus limits the application of this technique to loci, where there is no gene dosage effect. A null allele of the target gene in a heterozygous configuration should have no phenotype.

Independently of the strategy used to generate the regulated Cre expressing mouse strain, the offspring after crossing it to the reporter lacZ mouse should be carefully analysed, since a short ectopic expression of cre early in development could still be sufficient to induce recombination in a non-cell-type specific manner.

The limitations of cell type specific gene targeting are, firstly, the possible embryonic lethality or severe early post-natal phenotype which would abolish the analysis of gene function at later stages of development and, secondly, the possible complex scenario of the phenotype: throughout ontogeny cells in which site specific recombination took place are deficient for the product of the disrupted gene, which could lead to compensatory effects. Thus there could be either no detectable phenotype, or primary phenotypic effects are influenced by secondary compensatory effects.

Inducible gene targeting

The strategy of inducible gene targeting should be able to overcome these limitations since temporally regulated recombination should permit study of a phenotype before and after inactivation of the target

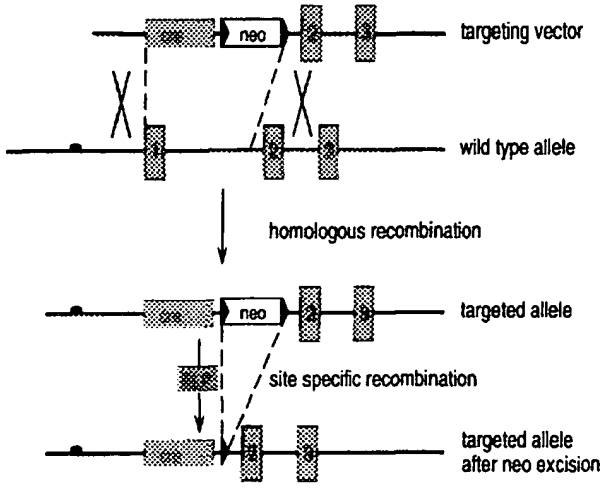

Figure 6. Targeted insertion of cre gene by gene replacement strategy. The first exon of the target gene is replaced by the coding region of Cre recombinase by keeping the position of the translational start codon unchanged. A neo gene in this case flanked by FRT sites (triangles) is inserted downstream of cre. The FRT flanked neo gene should be removed from the targeted ES cells by transient transfection with a plasmid encoding for the FLP recombinase.

gene at any given timepoint during ontogeny, even in the same individual mouse (Figure 7).

One promoter which has been used for this application is the interferon-alpha (IFN-α) inducible promoter of the mouse Mx1 gene. The Mx1 gene product is involved in defence reactions to viral infections and is silent in non-infected mice. IFN-α or IFN-β can transiently activate to high amount of transcription from the Mx1 promoter. *In vivo* induction can be achieved by using pIpC (synthetic double stranded RNA) which acts as an IFN inducer.

In this experiment transgenic mice for Mx-Cre were crossed to a mouse strain bearing a floxed allele of the β-polymerase gene (Figure 8). Cre mediated deletion in various tissues was quantified by Southern blot analysis of genomic DNA (Kühn *et al.* 1995).

The transgenic strain Mx-cre31a showed low background recombination in the absence of induction, but extensive deletion after induction by treatment with IFN or pIpC.

The heterogeneity in induced Cre-mediated recombination in the different tissues may reflect differing proportions of IFN responsive cells or IFN availability in various tissues.

Inducible and cell type-specific gene targeting

Since the use of the IFN inducible promoter of the Mx gene for inducible site specific recombination in mice is restricted for application to certain tissues and as there are not many other inducible promoters regulated tightly enough for this application in mice an alternative approach is being developed by the group of Francis Stewart at EMBL.

This approach, which allows the combination of tissue specific expression of Cre recombinase and the inducible regulation of its activity, is being developed for use in animals.

The inducibility of Cre can be achieved either by putting the recombinase gene under the control of an

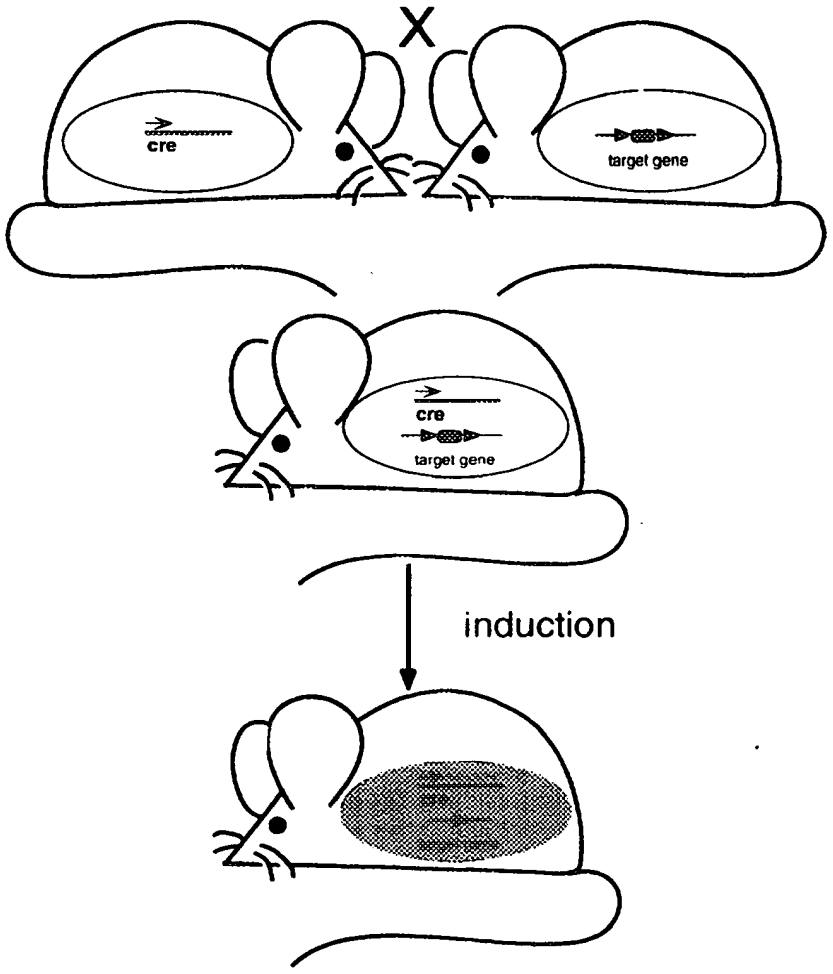

Figure 7. Breeding scheme for the approach of inducible gene targeting by homologous and site specific recombination. Only one allele of the target gene is shown. Two independent mouse strains have to be generated. One mouse strain should express the recombinase and is generated by conventional transgenesis. The cells which do express Cre upon induction are represented by the filled ellipse, cells that do not express Cre are shown as open white ellipses. The second mouse strain carried the gene of interest flanked by loxP sites in all cells. After intercross of the two strains inducible expression of the recombinase should lead to recombination in all cells accessible by the inducing ligand, indicated by the filled ellipse.

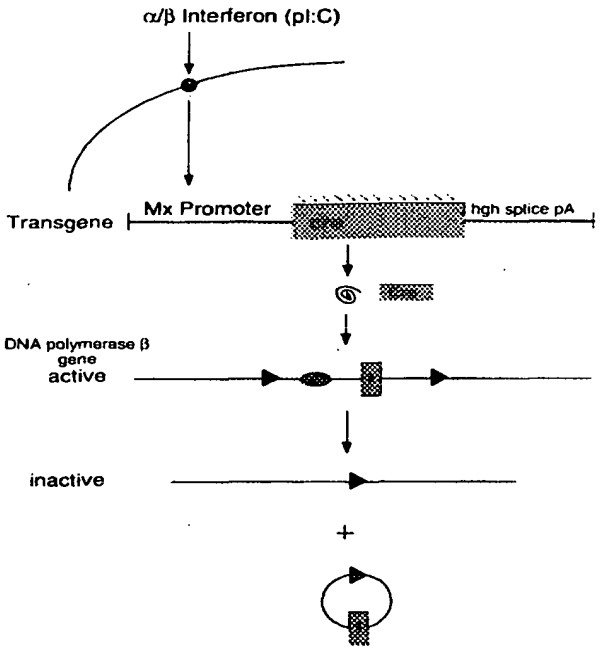

Figure 8. Inducible gene inactivation using the interferon (IFN) inducible promoter of the mouse Mx1 gene for the controlled expression of Cre recombinase. At the 3′ end of the transgene the splice donor and acceptor sites from the human growth hormone gene and a polyadenylation signal are located. The target sites for the Cre recombinase (loxP) are flanking the promoter region and the first exon of the mouse gene encoding for the DNA polymerase β. Only one allele of the target gene is shown. After activated expression of the Cre recombinase (small arrows) the DNA segment located between the two loxP sites is removed leading to an inactivation of the DNA polymerase β gene.

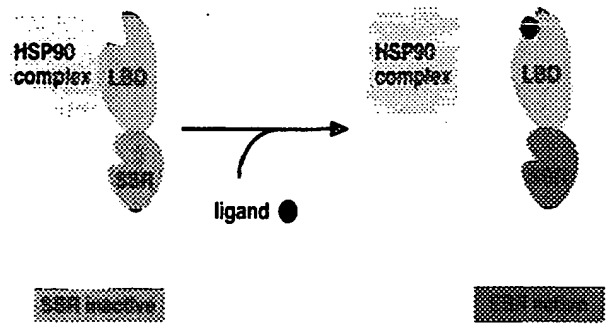

Figure 9. Ligand dependent activation of SSR (site specific recombinase)/LBD (steroid hormone receptor ligand binding domain) fusion proteins. The SSR/LBD is inactive in the absence of ligand (black filled dot) and active in the presence of ligand. Inactivation is probably mediated by association of the non-ligand bound LBD with an abundant protein complex containing HSP90 (heat shock protein). Ligand binding releases the receptor from the inhibitory complex.

inducible promoter or by the inducible regulation of its activity.

The latter can be achieved by creating a fusion protein between the recombinase and a ligand binding domain (LBD) from a steroid hormone receptor (Picard 1993). Such a fusion protein is inactive in the absence of ligand (hormone) and active in the presence of hormone (Figure 9). Inactivation is probably mediated by association of the non-ligand bound LBD with an abundant protein complex containing HSP90 (heat shock protein). Ligand binding releases the receptor from the inhibitory complex.

To screen for optimal inducible fusion partners an ES cell screening assay was developed (Figure 10). It consists of two randomly integrated transgenes. From one transgene the SSR/LBD fusion protein is expressed. The other transgene is a site specific recombination reporter construct which contains the previously described lacZ reporter cassette (Figure 5). For the selection in ES cells a second selectable marker gene (sm2) has been located between the two loxP sites. The SSR/LBD fusion protein is expressed constitutively. Upon hormone binding the SSR/LBD protein will be activated and excise the second selectable marker gene flanked by target sites. In this assay system it leads to activation of the lacZ reporter gene (Angrand *et al.* 1996; Kellendonk *et al.* 1996; Zhang *et al.* 1996).

For application in animals it is crucial that endogenous hormones do not function as a ligand for the SSR/LBD fusion protein. It is therefore necessary to work with ligand binding domains from mutated receptors, which can be activated only by synthetic steroids. The amount of synthetic steroid needed for the activation of recombination should be low enough that the side-effects the drug may have on the endogenous steroid hormone receptors is negligible and does not interfere with the analysis of the phenotype.

Comparison of the results in all three publications reveal the following scenario: the higher the activity of the SSR/LBD fusion protein, which can be induced by ligand, the higher seems to be the background level of activation of site specific recombination. It remains to be investigated how tight the regulation has to be for application in mice. Another parameter for use in animals would be the power of the promoter by which the expression of the fusion protein would then be controlled.

Combining cell type specific expression of the Cre recombinase with the conditional control of its activity, the use of SSR/LBD fusion proteins would allow both

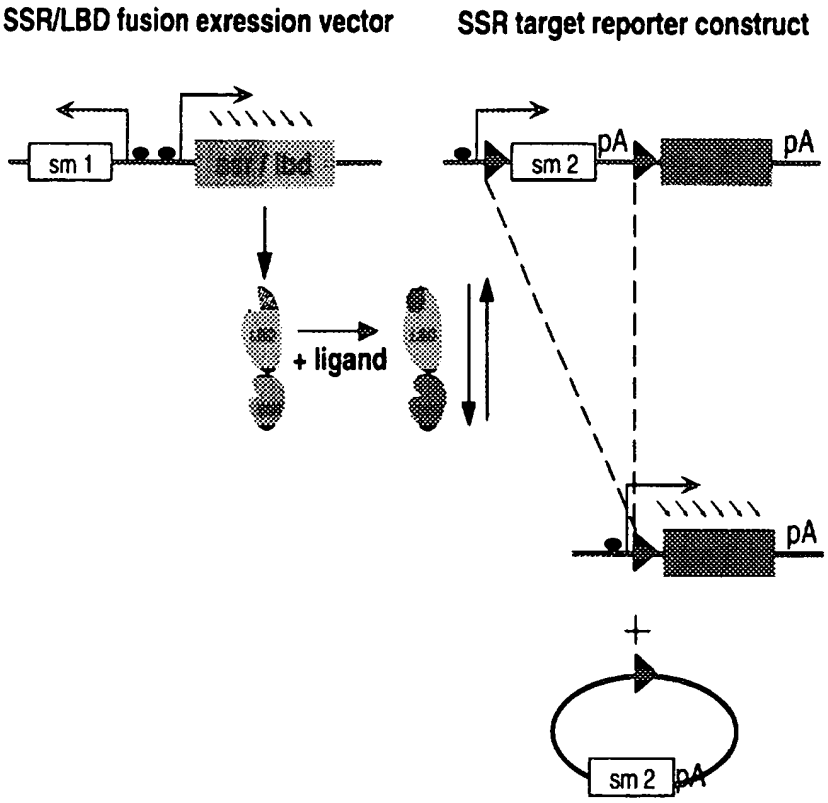

Figure 10. Inducible gene activation in ES cells mediated by SSR (site specific recombinase)/LBD (steroid hormone receptor ligand binding domain) fusion proteins. Two transgenes are necessary for the SSR/LBD inducible expression system. The SSR/LBD expression vector on the left side was integrated in the genome using the first selectable marker (sml). The site specific recombination reporter construct which contains the previously described lacZ reporter cassette (Figure 5) is shown on the right. For the selection in ES cells a second selectable marker gene (sm2) has been located between the two loxP sites. The SSR/LBD fusion protein is expressed constitutively (small arrows). Upon hormone binding the SSR/LBD protein will be activated and excise the second selectable marker gene flanked by target sites. This leads to activation of the lacZ reporter gene (small arrows). The big arrows indicate the location of the transcription start points.

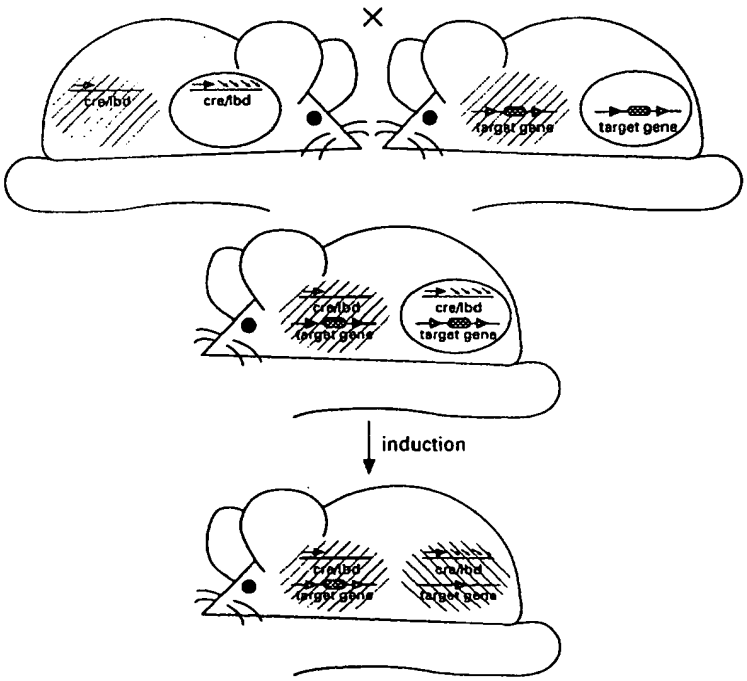

Figure 11. Breeding scheme for the approach of combined cell type-specific and inducible gene targeting by homologous and site specific recombination. Only one allele of the target gene is shown. Two independent mouse strains have to be generated. One mouse strain should express the recombinase in a cell type specific fashion and can be generated either by conventional transgenesis or by targeted insertion of the gene encoding the SSR/LBD fusion protein into a locus of interest. Cells which will not express SSR/LBD are represented by the ▨ filled circle, cells that will express SSR/LBD are shown as open white circles. The second mouse strain carries the gene of interest flanked by loxP sites in all cells. After intercross of the two strains cell type-specific expression and inducible activation of the SSR/LBD fusion protein should induce recombination in a particular set of cells, indicated by the ▧ filled circle.

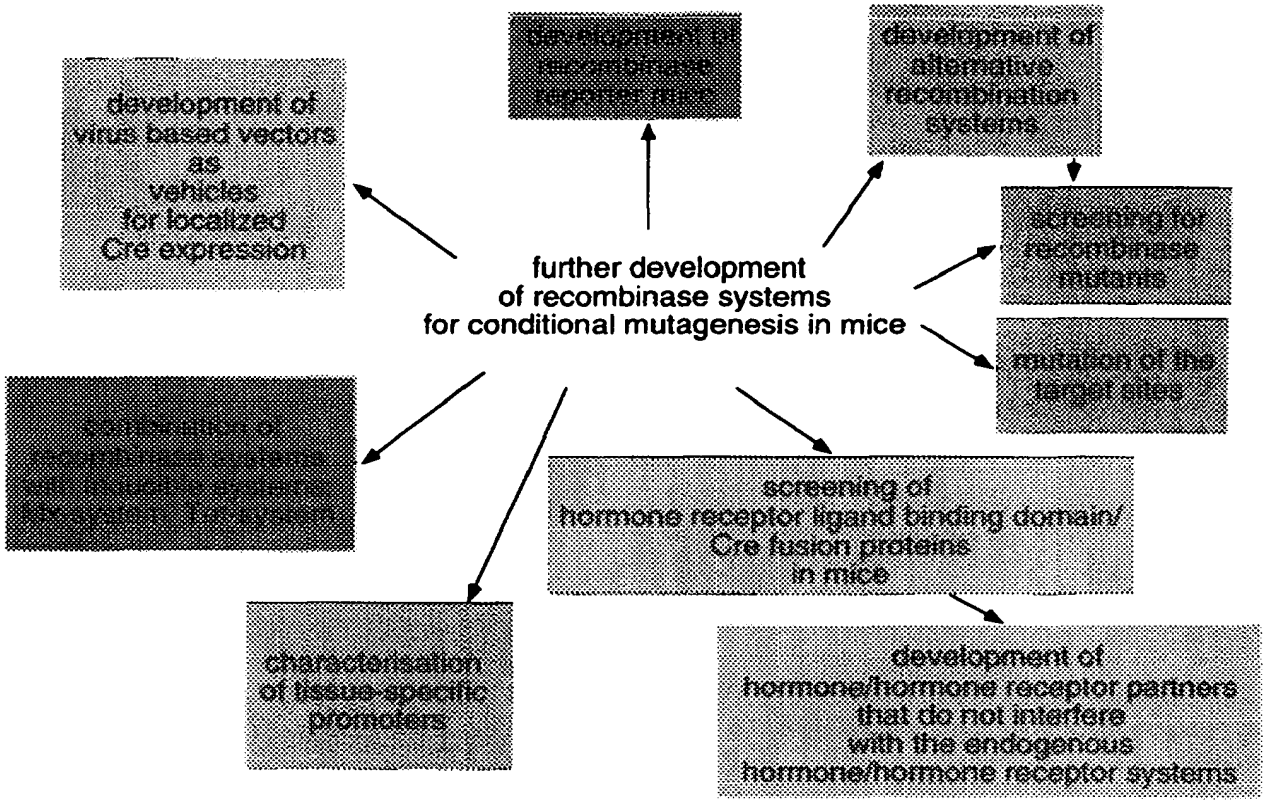

Figure 12. Future development of recombinase systems for conditional mutagenesis in mice.

spatial and temporal control of gene expression in either mouse embryos or the adult animal (Figure 11).

A number of directions for further development of recombinase systems for conditional mutagenesis in mice are outlined in Figure 12. The availability of more sophisticated technical support will catalyse the analysis of gene function and promote the mouse as a model system in various fields of biomedical research.

Gene targeting by homologous recombination in murine embryonic stem (ES) cells was first reported by Thomas and Capecchi (1987). Since then the analysis of gene function in the mouse by generating a number of mutant strains has made impressive progress. To introduce more subtle mutations or to generate gain-of-function mutants the site specific recombination system of the bacteriophage P1 has been developed as a tool for manipulating DNA *in vitro* and *in vivo*.

The characteristics of this recombinase system have been discussed here and its application to the genetic manipulation of the mouse genome reviewed. Further developments to achieve inducible gene targeting *in vivo* and *in vitro* were presented. Current work in the group led by Francis Stewart (unpublished) on the development of an inducible form of Cre, by generating a fusion protein with the ligand binding domains (LBDs) of steroid hormone receptors, has been summarized.

Thus the scientific questions which can be addressed by further development of the Cre/loxP recombination system are stimulating novel forms of genetic analyses in the mouse.

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